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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The overall goal of this grant has been to study the mechanisms by which non-genotoxic chemicals act. Specifically, the working hypothesis has been that chemical modulation of gap junctions could lead to many toxic endpoints, such as teratogenesis, tumor promotion, immune-, reproductive- and neuro-toxicities. To test this hypothesis, we set up several aims: (a) to develop new methods to measure gap junction function (Fluorescence Recovery After Photo-bleaching and scrape-loading/dye transfer); (b) to test if several known model non-genotoxic chemicals inhibit intercellular communication in several cell types; and (c) to study the biochemical mechanisms by which various chemicals inhibit intercellular communication. Results of this 3 year study have produced (a) three new validated in vitro methods to measure gap junction function; (b) produced overwhelming evidence that known non-genotoxic teratogens, tumor promoters, neuro-, immune- and reproductive-toxicants can inhibit gap junction function; (c) evidence suggesting several biochemical mechanisms by which these chemicals act; and (d) helped develop a new theoretical framework for a biologically-based risk assessment.				
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assessment model system. Multiple peer-reviewed articles, book chapters, abstracts and symposium talks have been generated by this grant.

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**AFOSR-TR. 89-0455**  
**FINAL TECHNICAL REPORT**

**AFOSR-86-0084**

2/25/86 to 2/28/89

**"The Role of Chemical Inhibition of Gap Junctional  
Intercellular Communication in Toxicology"**

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## I. SUMMARY

One of the major challenges in the field of toxicology is to be able to predict risk of exposure to various chemicals. The whole field of "risk assessment" has been hampered by the limitations of animal bioassay results, by the unavailability of reliable short-term assays to detect the three primary biological endpoints of cells, namely mutagenic, cytotoxic and epigenetic effects.

To the objective of understanding how non-genetic toxic chemicals, which are known teratogens, tumor promoters, neurotoxicants, reproductive- and immune-toxicants, work, we proposed to test the hypothesis that inhibition of gap junctional intercellular communication might lead to these aforementioned toxic endpoints. The rationale for this hypothesis was that, since gap junction function is a fundamental biological process needed to maintain tissue homeostasis and control of cell growth and differentiation, alteration of this function would have mal-consequences on the organism level.

During these three years we have (1) validated the "Fluorescence Recovery After Photobleaching" or "FRAP analysis" technique to measure gap junction function; (2) developed and validated a scrape-loading/dye transfer technique; (3) developed a cell-sorter/dye transfer to measure cell communication; (4) integrated the "FRAP" technique to the scrape-loading/dye transfer technique for single cell quantitation; (5) tested several known and model tumor promoters, neurotoxicants, immune- and reproductive-toxicants (i.e., toxaphene, DDT, polybrominated biphenyls, dieldrin, phenobarbital) and showed they all inhibited intercellular communication in vitro; (6) demonstrated cell type, tissue and species specificity to chemical inhibition of intercellular communication; (7) clearly established multiple biochemical mechanisms by which various chemical inhibitors of intercellular communication work, in particular, the phorbol ester works by activating protein kinase C; (8) developed a new in vitro human keratinocyte assay to test potential human skin tumor promoters; (9) initiated studies linking certain oncogenes with chemical inhibitors of intercellular communication; and (10) introduced new scientific concepts for the new field of epigenetic toxicology and theoretical basis for a biologically-based cancer risk assessment model for exposure to chemicals.

New work which will be continued in the next grant will be to further delineated how various biochemical mechanisms act to inhibit gap junction function. New mutant cells which affect gap junction function/structure will be analyzed.

## II. RESEARCH OBJECTIVES

The original objective of this grant was to examine if the inhibition of gap junctional intercellular communication was a

mechanism by which non-genotoxic chemicals acted to induce teratogenic, tumor promoting, neurotoxicity, immune modulation or reproductive dysfunction. We were able in three years to stick to these objectives, since all of our specific aims became doable. Those aims included: (a) development and validation of new in vitro techniques to measure gap junction function; (b) test if known toxicants could inhibit intercellular communication; (c) examine various potential biochemical mechanisms by which various chemical toxicants could inhibit intercellular communication; and (d) to study gap junction function with the use of antibodies to gap junction proteins, isolation of genetic mutants for intercellular communication and various oncogenes known to interfere with gap junction function.

### III. SUMMARY OR RESEARCH

After three years of research, we believe we have achieved successful testing of four of the five specific aims, as measured by the number of peer-reviewed articles, requested review/book chapters, and multiple invited symposium and seminar opportunities (see Section of Published Manuscripts). Moreover, the culmination of this effort came in the form of an international symposium which I organized at Michigan State University on September 28-30, 1988. The topic, "Toxicological Implications of Altered Gap Junctional Intercellular Communication", was a first of its kind and drew investigators from the disciplines of toxicology, risk assessment and gap junction biology [publication of the symposium is forthcoming]. The most significant unforeseen consequences of this research is the conceptual and theoretical insight of the results of chemical inhibitors of intercellular communication have produced. This has led to the concept of "epigenetic toxicology" and to new ways of developing a biologically-based carcinogenic risk assessment model for exposures to chemicals. In addition, the significance of these findings impacts on the use of in vitro assays to eliminate the use of many animal toxicity.

The following abstracts from our published results over the 1st few years concisely summarizes results related to our original objective and the specific aims.

Aim 1. To develop and validate/characterize new in vitro assays to measure intercellular communication.

A. Flow Cytometry and Scrape-loading/Dye Transfer as a Rapid Quantitative Measure of Intercellular Communication in Vitro.

Terrance J. Kavanagh, George M. Martin, Mohamed H. El-Fouly, James E. Trosko, Chia-Cheng Chang, and Peter S. Rabinovitch.

We describe two flow cytometric assays

performed on populations of cells which have been stained with various fluorescent tracer molecules by the scrape-loading technique. One assay uses a simple one-color analysis on a flow cytometer by quantitating the fluorescence intensity of scrape-loaded lucifer yellow CH (LY) in individual cells. The other assay utilizes a two-color analysis on a cell sorter whereby cells which are initially loaded (donors) are identified by their uptake of both rhodamine isothiocyanate-e\dextran and LY, whereas the recipients of dye transfer are identified as having LY only. Agents which have been shown to inhibit intercellular communication in other assays exhibit similar blocking activity in LY transfer and this is readily quantitated by flow cytometry. The two-color analysis has the added advantage of being able to identify both donors and recipients.

#### B. Scrape-Loading and Dye Transfer

Mohamed H. El-Fouly, James E. Trosko, and Chia-Cheng Chang.

Gap junction-mediated intercellular communication has been recognized in cells from different tissues of various organisms and has been implicated in a variety of cellular functions and dysfunctions. Here we describe a new, direct and rapid technique with which to study this cellular phenomenon. It employs scrape-loading to introduce a low molecular weight (MW) fluorescent dye. Lucifer yellow CH (MW 457.2) into cells in culture and allows the monitoring of its transfer into contiguous cells. In communication-competent cells the dye transmission occurred within minutes after loading. The involvement of membrane junctions in Lucifer yellow transfer was verified by the concurrent loading of a high MW marker dye conjugate, rhodamine dextran (MW 10,000). Once introduced intracellularly the rhodamine dextran is unable to cross the relatively narrow membrane junctions. Chemicals of variable potency known to block junctional communication were tested in Chinese hamster V79 cells and other mammalian cells. The results showed effective blockage of the dye transfer at non-cytotoxic doses. This new technique can be applied to a wide variety of

mammalian (including human) cells. In addition, it has the potential to be utilized as a rapid screening assay to detect chemicals that can modulate intercellular communication and to study their mechanism of action.

- C. Anchored Cell Analysis/Sorting Coupled with the Scrape-Loading/Dye Transfer Technique to Quantify Inhibition of Gap-Junctional Intercellular Communication in WB-F344 Cells By 2,2',4,4',5,5'-Hexabromobiphenyl.

Mark G. Evans, Mohamed H. El-Fouly, James E. Trosko, and Stuart D. Sleight.

Inhibition of intercellular communication has been hypothesized to play a role in tumor promotion. The compound 2,2',4,4',5,5'-hexabromobiphenyl (245-HBB) is a tumor promoter in vivo and blocks intercellular communication in vitro. The scrape-loading/dye transfer (SL/DT) assay was used to assess this in vitro effect at varying concentrations of 245-HBB. The SL/DT technique is based on the intracellular loading of a fluorescent dye, lucifer yellow (LY), and monitoring its transfer into adjacent cell's via patent gap junctions. Confluent WB-F344 (rat epithelial) cells were exposed to various noncytotoxic concentrations of 245-HBB. Transfer of LY was then quantified with anchored cell analysis/sorting (ACAS-470, Meridian Instruments, Okemos, Mich.). The results indicate an inverse correlation between the degree of fluorescence in secondary LY-recipient cells and the treatment concentration. The coupling of these two methods of cellular biology provided rapid quantitative analysis of dye transfer in measuring the concentration/response of modulation of gap-junctional permeability in cultured cells.

- D. Concentration/Response Effect of 2,2',4,4',5,5'-Hexabromobiphenyl on Cell-Cell Communication In Vitro: Assessment by Fluorescence Redistribution After Photobleaching ("FRAP").

Mark G. Evans, and James E. Trosko.

Inhibition of gap junctional-mediated cell-cell communication might be a mechanism for several

types of cellular dysfunctions, including tumor promotion. Although many different assays have been designed to measure gap junction-mediated intercellular communication, we applied a new technique, termed Fluorescence Redistribution After Photobleaching ("FRAP"), to assess the ability of a known tumor promoter, 2,2',4,4',5' 5-hexabromobiphenyl (245-HBB), to inhibit cell-cell communication in a concentration-dependent manner. WB-F344 (rat epithelial) cells were plated at low density, exposed to noncytotoxic concentrations of 1, 5, or 20  $\mu$ g 245-HBB/ml medium, and stained with 6-carboxyfluorescein diacetate. Single cells in pairs or clusters of touching cells in each exposure group were examined with FRAP. The results revealed an inverse correlation between the degree of fluorescence redistribution in photobleached cells and the concentration of 245-HBB. Therefore, FRAP appears to be a sensitive and rapid technique for determining complete or partial inhibition of chemically induced intercellular communication *in vitro*. These results also provide further evidence for the ability of 245-HBB to inhibit gap junction-mediated cell-cell communication in a concentration-dependent manner.

E. Altered Regulation of Intercellular Communication by Epidermal Growth Factor, Transforming Growth Factor- $\beta$  and Peptide Hormones in Normal Human Keratinocytes

B.V. Madhukar, S.Y. Oh, C.C. Chang, M. Wade and J.E. Trosko.

Since many chemical tumor promoters and some oncogenes have been shown to inhibit gap junction-mediated intercellular communication, the effect of various growth factors on gap junctional intercellular communication on normal human keratinocytes was examined. In order to measure the effect of the growth factors on gap junctional communication, the scrape-loading/dye transfer technique was used on human keratinocytes placed in serum-free medium *in vitro*. At 24 h after treatment epidermal growth factor (10 ng/ml), transforming growth factor- $\beta$  (1 ng/ml), whole bovine pituitary extract (70  $\mu$ g/ml) and 12-O-tetradecanoyl-phorbol-13-acetate (TPA) (100 ng/ml) inhibited intercellular communication.



Treatment of these cells with transforming growth factor- $\beta$  (1 ng/ml) induced morphological changes in some of the cells and brought about selective intercellular communication within and between the nonaltered and altered cells. Epidermal growth factor and whole bovine pituitary extract, significantly enhanced [ $^3$ H]thymidine uptake and also stimulated cellular proliferation under the experimental conditions used to inhibit intercellular communication. Both transforming growth factor- $\beta$  and TPA markedly inhibited [ $^3$ H]thymidine uptake and induced differentiation of some of these cells. In order to study the possible mechanism by which the growth factors might inhibit intercellular communication, the effect of the growth factors on protein kinase C activation and alterations of intracellular free calcium was investigated. The results indicated that neither protein kinase C nor an increase in  $[Ca^{2+}]_i$  were involved in the modulation of gap junctional communication by epidermal growth factor or transforming growth factor- $\beta$ . The study suggests that in the human keratinocytes inhibition of intercellular communication may be involved (i) in the action of growth factors such as epidermal growth factor during cellular proliferation and (ii) in the differentiation of primary keratinocytes by transforming growth factor- $\beta$ .

F. Dieldrin Inhibition of Junctional Intercellular Communication in Rat Glial Cells as Measured by the Fluorescence Photobleaching and Scrape Loading/Dye Transfer Assays.

S. Suter, J.E. Tresko, M.H. El-Fouly, L.R. Lockwood, and A. Koestner.

Application of the fluorescence-recovery after photobleaching (FRAP analysis) technique and scrape loading/dye transfer assay was made to measure the presence of gap junctional communication in primary rat glial cells in vitro in the presence and absence of the neurotoxicant and tumor promoter dieldrin, a chlorinated insecticide. Results demonstrate that primary rat glial cells are able to exhibit gap junctional intercellular communication and that dieldrin at noncytotoxic

concentrations can modulate gap junctional communication as early as 10 min after exposure to the chemical and that the effect is reversible after 4 hr recovery from the dieldrin exposure. Both the FRAP analysis and the scrape-loading/dye transfer assay have validated the observation that dieldrin inhibits gap junctional communication in other cell types using different techniques to measure gap junction function. These results were interpreted as an indication that inhibition of gap junctional communication might contribute to the cellular mechanism of dieldrin's neurotoxicity.

Aim 2. To use model toxic, but non-genotoxic, chemicals as potential modulators of intercellular communication.

A. Inhibition of Gap Junctional-Mediated Intercellular Communication In Vitro By Aldrin, Dieldrin, and Toxaphene: A Possible Cellular Mechanism for Their Tumor-Promoting and Neurotoxic Effects

J.E. Trosko, C. Jone, and C.C. Chang.

Several mechanisms have been postulated to be responsible for the pleiotropic effects of toxic chemicals. Although the cytotoxicity and mutagenicity of chemicals are well studied and relatively easily detected, the noncytotoxic and nonmutagenic (i.e., epigenetic) mechanisms of chemical toxicity are less well understood. An in vitro assay, using cocultures of Chinese hamster cells to measure metabolic cooperation between V79 6-thioguanine-sensitive (6TG<sup>s</sup>) cells and resistant (6TG<sup>r</sup>) cells, has been developed to detect noncytotoxic and nonmutagenic chemicals that inhibit, quantitatively, gap junctional communication. The insecticides aldrin, dieldrin, and toxaphene, known to have pleiotropic toxic effects in animals, were shown to inhibit gap junctional communication. Interpretations of results suggests that chemical inhibition of gap junctional communication could be a possible mechanism to explain their tumor promoting and neurotoxic effects.

B. Effects of Hepatic Tumor Promoters Phenobarbital and Polybrominated Biphenyls on Intercellular Communication Between Rat Liver Epithelial Cells

M.S. Razabek, J.E. Trosko, C. Jone, and S.D. Sleight.

FireMaster BP-6 (FM), a mixture of polybrominated biphenyls, and phenobarbital (PB) promote hepatic carcinogenesis in rats. Inhibition of intercellular communication is a possible mechanism of tumor promotion. Vitamin A compounds, such as retinyl acetate (RA), antagonize the carcinogenic process in some systems. In this study, FM, PB and RA were tested in two intercellular communication assays using a rat liver epithelial cell line (WB-F344). One assay measured inhibition of metabolic cooperation (MC) between cells containing the enzyme hypoxanthine-guanine phosphoribosyl transferase and mutant cells lacking the enzyme. The other assay evaluated the inhibition of fluorescence redistribution after photobleaching (FRAP), which occurs through gap junctions between cells loaded with a fluorescent dye. The hepatic tumor promoter PB inhibited MC, but did not block junctional communication in the FRAP assay. The hepatic tumor promoter FM inhibited MC and also blocked FRAP. Retinyl acetate blocked MC but did not inhibit FRAP, and had no effect on the ability of FM to block junctional communication in the MC or FRAP assays.

Aim 3. To study the specific biochemical mechanisms by which various chemical inhibitors of intercellular communication work.

A. Inhibition of Gap Junctional Blockage by Palmitoyl Carnitine and TMB-8 in a Rat Liver Epithelial Cell Line

S.Y. Oh, B.V. Madhukar, and J.E. Trosko.

Exposure to 12-O-tetradecanoylphorbol-13-acetate (TPA) has been shown to inhibit gap junctional intercellular communication (GJIC) in many cell types in vitro. Using a scrape-loading/dye transfer technique, TPA was shown to cause a dose-dependent and transient inhibition of GJIC in WB-344, a normal rat

liver epithelial cell line. Such a down-modulation of intercellular communication was found to be associated with an increase in protein kinase C (PKC) activity. Translocation of this activity to the particulate fraction occurred 10 min after exposure to 16 nM TPA and was consistent with the time course needed to inhibit GJIC. After 6 h exposure to TPA, essentially all the PKC activity was lost concurrent with the recovery of communication in these cells. During this time, the cells also became refractory to inhibition by further addition of TPA. Blockage of communication induced by TPA in WB cells was prevented by treating the cells with 23 M palmitoyl carnitine for 1 h or 100 M 8-N-N-(diethylamino)-octyl-3,4,5-trimethoxybenzoate for 30 min. The results indicated that TPA transiently modulates GJIC in WB cells and PKC activation is possibly involved in blockage of communication in these cells.

B. Synergistic Inhibition of Metabolic Cooperation by Oleic Acid or 12-O-Tetradecanoylphorbol-13-acetate and Dichlorodiphenyltrichlorethane (DDT) in Chinese Hamster V79 Cells: Implication of a Role for Protein Kinase C in the Regulation of Gap Junctional Intercellular Communication

C.F. Aylsworth, J.E. Trosko, C.C. Chang, K. Benjamin, and E. Lockwood.

The effects of TPA and/or DDT and oleic acid and/or DDT on gap junction-mediated intercellular communication (i.e., metabolic cooperation) between Chinese hamster V79 cells was examined. Addition of TPA, DDT or oleic acid alone to cocultures of 6-thioguanine-resistant (6-TG<sup>r</sup>) and 6-thioguanine-sensitive (6-TG<sup>s</sup>) V79 cells significantly increased the recovery of 6-TG<sup>r</sup> cells indicating inhibition of metabolic cooperation. In the presence of TPA and DDT or oleic acid and DDT the observed recovery of 6-TG<sup>r</sup> cells was significantly greater than the expected (calculated) additive 6-TG<sup>r</sup> cell recovery. No synergistic increases in 6-TG<sup>r</sup> cell recovery were observed when cocultures of V79 cells were exposed to dieldrin and DDT. These results indicate that TPA and DDT or oleic acid and DDT can act

synergistically to inhibit metabolic cooperation. These data suggest a role for protein kinase C in the regulation of gap junction-mediated intercellular communication.

Aim 4. Use of antibodies to gap junction proteins, genetic mutants to gap junctional intercellular communication and various oncogenes to study the mechanisms by which gap junctions can be regulated and the biological consequences of that modulation.

This aim is still on-going and is the primary basis for the new USAFOSR grant. However, achievements were made.

A. Loss of Intercellular Junctional Communication Correlates with Metastatic Potential in Mammary Adenocarcinoma Cells

G.L. Nicolson, K.M. Dulski, and J.E. Trosko.

A series of rat 13762NF mammary adenocarcinoma cell sublines and clones of various spontaneous pulmonary metastatic potentials from the mammary fat pads of syngeneic rats were examined for their intercellular junctional communication. Using the scrape-loading/dye transfer technique to introduce Lucifer yellow (M, 457) into cells, we measured the abilities of 13762NF cells to transfer dye to adjacent cells. There was an excellent correlation between loss of Lucifer yellow dye transfer and spontaneous metastatic potential (average total volume of lung metastases inversely correlated to % cells coupled,  $r = 0.93$ ; average total number of lung metastases inversely correlated to % cells coupled,  $r = 0.91$ ). The data suggest that high metastatic potentials are closely correlated with loss of intercellular junctional communication in these malignant mammary tumor cells.

B. Characteristic of Some Mutants Lacking Gap Junction Communication

S.Y. Oh, B.V. Madhukar, C.C. Chang, J.E. Trosko and E.C. Beyer.

To understand the basic mechanism(s) regulating gap junction communication we have developed a series of gap junction communication deficient (GJIC) mutants in a normal rat liver

epithelial cell (WB-F344) which is hypoxanthine guanine phosphoribosyl transferase deficient (HGPRT<sup>-</sup>). The cells were exposed to a mutagenesis regime and cocultured with the wild type HGPRT<sup>+</sup> cells. A total of 83 metabolic cooperation deficient (MC<sup>-</sup>) clones were selected in the presence of 6-thioguanine and 20% of these clones were GJIC<sup>-</sup> mutant cells when screened by scrape-loading/dye transfer technique. All 16 clones, when examined further by fluorescence recovery after photobleaching (FRAP) analysis, were blocked in gap junction communication. Characterization of some of these mutants by phorbol ester binding studies indicated there was no difference in phorbol 12-myristate 13-acetate (PMA) binding between normal and communication deficient cells. This suggests that there was no elevation in membrane associated protein kinase C activity or intracellular diacylglycerol in these mutants. The mutants were further examined for the presence of gap junction protein using rat liver antibody.

C. Phenotypic Transformation and Inhibition of Gap Junctional Intercellular Communication in Epithelial and Mesenchymal Cells by the NEU Oncogene

M.H. El-Fouly, J.E. Trosko and C.C. Chang.

Carcinogenesis is a multistep process that seems to involve both genetic and epigenetic events which could lead to changes in membrane structure and function. Among the various membrane functions, direct gap junction-mediated intercellular communication (GJIC) is considered an important process by which contiguous cells regulate their growth and differentiation. Several tumor promoters and, recently, certain oncogene products, e.g. src and H-ras, and growth factors e.g. EGF, have been found to block GJIC. The neu oncogene is related to erb-B gene and encodes a 185,000 Mr protein (p185) similar to epidermal growth factor receptor. The p185 is a transmembrane polypeptide with a tyrosine kinase domain that may represent a receptor for an unidentified growth factor. Its amplification and overexpression correlate with advanced stage

and metastasis of epithelial tumors. We introduced the neu oncogene, with a neomycin resistance marker gene, in a primary epithelial rat liver cell and in rat glial cell line and tested their ability to conduct GJIC. Transfectants were selected in G418 and 20 separate colonies from each cell type, isolated and assayed by the Scrape-Loading/Dye Transfer technique. All colonies with G418 resistance showed phenotypic transformation, contact insensitivity and vertical aggregation in culture. These cells, when compared to controls, showed remarkable inhibition of GJIC. This association between transformation by neu oncogene and cellular uncoupling implies a role of p180 in the inhibition of GJIC which might represent an essential step in transformation and possibly progression and metastasis during carcinogenesis.

Aim 5. Mechanistic Basis for "EPIGENETIC TOXICOLOGY" and a Biologically-based Risk Assessment Model.

A. Nongenotoxic Mechanisms in Carcinogenesis:  
Role of Inhibited Intercellular Communication

J.E. Trosko and C.C. Chang.

Mutagenesis Is Carcinogenesis: A Failed Paradigm.

Raw observations in science must be interpreted to be understood. In all scientific disciplines, the prevailing paradigm helps the scientific community to interpret the results of experiments. In cancer research, the paradigms, "carcinogens are mutagens" (Ames et al. 1973) and "genotoxicity" (Ehrenberg et al. 1973) have shaped most of the present thinking in the area of understanding the mechanism(s) of carcinogenesis and the practical issue of risk assessment to human beings to cancer after exposure to radiation and chemical agents. Although we wish to make it clear we feel mutagenesis does play a significant role in carcinogenesis, as is evident from genetic predispositions to human cancer (Trosko et al. 1985) and from all the evidence that mutagens and mutations found in experimental studies on mammalian and human cells (somatic mutation theory of cancer; see [Trosko and Chang 1978]), we must emphasize that carcinogenesis is more than mutagenesis. These are two independent

and nonequivalent biological processes, one taking place in an organism (the former), whereas the other takes place within a single cell.

The multistep nature of the cancer process has been noted, both in experimental animals and in the normal clinical course of tumor development in human beings (Foulds 1975; Nowell 1976; Cairns 1981). Conceptually, the initiation, promotion, and progression stages have been developed to explain the multistep nature of carcinogenesis (Boutwell 1974; Pitot et al. 1981). Clearly, these operational concepts on the whole animal level do not imply any specific mechanism, nor is it yet known what the mechanism(s) is (are) that underlie each of these steps. However, one thing appears perfectly clear, initiation and promotion are two distinct processes, and, therefore, the underlying cellular and molecular mechanisms must be different (Trosko et al. 1983a). Several working hypotheses have been advanced, suggesting that initiation, an irreversible process, involves a mutagenic event, promotion involves a mitogenic event (Trosko and Chang 1983), whereas progression might involve another event, possibly requiring either another mutagenic hit (Trosko and Chang 1980; Potter 1981; Trosko and Chang 1986) and/or another epigenetic process (Frost and Kerbel 1983). Therefore, the objective of this report is to suggest that biological data, related to the multistep carcinogenic process, must be incorporated into any risk assessment process and that the current paradigm shaping the design of the bioassay and of short-term assays for carcinogen testing must be challenged to include the nongenotoxic properties of chemicals that could influence the ultimate appearance of cancers in humans (Trosko and Chang 1985).

B. Chemical and Oncogene Modulation of Gap Junctional Intercellular Communication

J.E. Trosko and C.C. Chang.

Crisis in Toxicology: Inadequacy of the Concept of Genotoxicity.

Obviously, exposure to chemicals can lead to both beneficial/adaptive and harmful/maladaptive responses at the whole



organism level. At the cell level, the chemical could (a) alter the genetic information [mutagenicity]; (b) induce cell death by a wide variety of mechanisms (e.g., lethal mutations, enzyme inhibition, membrane destruction) [cytotoxicity]; and/or (c) modulate gene expression [epigenetic modulation]. Depending on a wide variety of factors, such as the stage of development (early embryonic versus adult stage); how many cells have been affected; concentration of the chemical; genetic defense mechanisms of the organism; synergisms or antagonisms with other endogenous or exogenous chemicals; how many and which genes might be affected; whether the affected cell is clonally amplified or not, etc.], the biological consequences of this mutagenic, cytotoxic or epigenetic change could vary from the undetectable, to acute toxic reactions and death, to various chronic diseases.

In recent years, one of the most powerful paradigms to study chemical toxicity has been the introduction of the concept of "genotoxicity". Clearly, since the fidelity of the genetic information in both germ and somatic cells are needed for maintenance of all levels of the biological hierarchy in multicellular organisms, induction of gene and chromosomal mutations are known or suspected to be associated with a wide spectrum of genetic and somatic diseases. Therefore, to study mutagenesis and to detect environmental mutagens have been, and will continue to be, an important element of toxicology.

Unfortunately, this paradigm, as important as it is, has blinded us to other mechanisms by which chemicals could be toxic to organisms. In other words, while it is true and clear that mutagens can be harmful, not all harmful chemicals are mutagenic!. In this report, we will present another paradigm, namely chemical modulation of gap junctional intercellular communication as a cellular mechanism of chemical toxicity which could lead to a wide variety of harmful endpoints. Specifically, we and others have postulated that chemical modulation of gap junctional communication can lead to teratogenesis, promote initiated cells during carcinogenesis, cause neurotoxic effects, bring about reproductive dysfunction and other dysfunctional physiological states.

#### IV. LIST OF PUBLISHED MANUSCRIPTS AND PREPRINTS

S. Suter, J.E. Trosko, and A. Koestner, "Fluorescence photobleaching assay of dieldrin inhibition of gap junctional intercellular communication in rat glial cells." Fund. Appl. Toxicol., 9:785-794, 1987.

J.E. Trosko, C. Jone, and C.C. Chang, "Inhibition of gap-junctional-mediated intercellular communication, in vitro, by aldrin, dieldrin and toxaphene: A possible cellular mechanism for their tumor-promoting and neurotoxic effects." Molecular Toxicol., 1:83-93, 1987.

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#### V. PROFESSIONAL PERSONNEL

J.E. Trosko, Ph.D., Professor of Pediatrics/Human Development, College of Human Medicine, Center for Environmental Toxicology, Michigan State University, Principal Investigator.

B.V. Madhukar, Ph.D., Assistant Research Professor, Department of Pediatrics/Human Development.

M.H. El-Fouly, Graduate Student, M.D. from University of Alexandria, Egypt and M.S. from University of Michigan, 1984. He received his Ph.D., Winter term, 1988.

Yuh Shan Jou, Graduate Student from Taiwan, is a Ph.D. Candidate in Genetics.

Saw Yin Oh, Ph.D., Research Associate, Department of Pediatrics/Human Development, (Replaced Laurie Parker). Has now returned to Australia.

## VI. SPOKEN PAPERS

M.H. El-Fouly and J.E. Trosko, "Scrape-loading and dye transfer: A rapid and simple technique for the detection of intercellular communication." Tissue Culture Assoc. Mtg., Chicago, June 4-8, 1986.

J.E. Trosko, C.C. Chang, M.H. El-Fouly, R. Kulkarni, and R. Gera, "Role of gap junctional communication in normal and malignant human epithelial cells." Intern. Cell and Tissue Culture Confer., Hershey, PA, Sept. 25, 1986.

M.H. El-Fouly, S.T. Warren, J.E. Trosko, and C.C. Chang, "Inhibition of gap junction-mediated intercellular communication in cells transfected with the human H-ras oncogene." Amer. Soc. Human Genetics Mtg., Philadelphia, PA, November 2-5, 1986.

J.E. Trosko (seminar), "Adaptive and nonadaptive consequences of chemical inhibition of intercellular communication." Columbia University College of Physicians and Surgeons, April 7, 1986. [Host, Dr. Carmia Borek].

J.E. Trosko (seminar), "Oncogenes, intercellular communication and carcinogenesis." Dept. Pathology, New York University Medical Center, April 8, 1986. [Host, Dr. Angel Pellicer].

J.E. Trosko (seminar/consultant), "New methods to detect chemical inhibitors of intercellular communication." R.J. Reynolds/Nabisco Laboratory, Winston-Salem, NC, May, 1986. [Host, Dr. Dave Doolittle].

M.H. El-Fouly and J.E. Trosko (poster), "Role of chemical inhibition of cell-cell communication in toxicology." July 31, 1986. [Organizer, Dr. Steve Aust].

J.E. Trosko, "Chemical and oncogene modulation of gap junctional intercellular communication." NIEHS Conference, "Tumor Promoters: Biological approaches for mechanistic studies and assay systems." Research Triangle Park, NC, Sept. 8-10, 1986. [Organizer, Dr. R. Lagenbach].

J.E. Trosko (symposium speaker), "The role of inhibition of DNA polymerase  $\alpha$  in DNA amplification in Chinese hamster cells." Deutscher Krebsforschungs-zentrum, Heidelberg, Germany, Oct. 24-26, 1986.

J.E. Trosko (symposium talk), "Role of intercellular communication on aging." University-Based Research on Aging, Michigan State University, Nov. 11, 1986.

J.E. Trosko (seminar speaker), "Pharmacological and toxicological effects of chemical modulation of gap junction function." Dept. Pharmacology and Toxicology, M.S.U., Nov. 13, 1986.

J.E. Trosko (seminar speaker), "Inhibition of gap junctional communication by chemicals and oncogenes during carcinogenesis." Boston University School of Medicine, Dec. 4, 1986.

J.E. Trosko (seminar speaker), "Oncogenes, inhibition of intercellular communication and tumor promotion." Emory University School of Medicine, Atlanta, Dec. 12, 1986.

J.E. Trosko (seminar speaker), "Oncogenes, inhibition of intercellular communication and tumor promotion: An integrated hypothesis involving inhibition of gap junctional communication." University of Texas Cancer Center, Science Park, Texas, April 14, 1987.

J.E. Trosko (symposium speaker), "Oncogenes, growth factor and tumor promoter modulation of gap junctional communication." International Conference on Gap Junctions, Asilomar Conference Center, Pacific Grove, California, July 6-10, 1987.

J.E. Trosko (symposium speaker), "Non-genotoxic mechanisms in carcinogenesis: Role of inhibited intercellular communication." Banbury Conference, Cold Spring Harbor, NY, Oct. 11, 1987.

J.E. Trosko (symposium speaker), "Chemical modulation of gap junctional communication: Implications for varied toxicological responses." Northeast Regional Society of Toxicology Mtg., Boston, Oct. 23, 1987.

J.E. Trosko (lecturer), "Oncogenes, chemical tumor promoters and growth factors: An integrated hypothesis for carcinogenesis." Univ. of Maryland School of Medicine, Dept. of Pathology, Nov. 12, 1987.

J.E. Trosko (seminar speaker), "Oncogenes, chemical tumor promoters and gap junctions: An integrated theory of carcinogenesis." Frederick Cancer Res. Facility, Dec. 9, 1987.

J.E. Trosko (invited speaker), "Towards understanding carcinogenic hazards: A crisis in paradigms." Brookings Institute, Wash., D.C., Jan. 12, 1988.

J.E. Trosko (course lecturer), "Role of chemical inhibition of intercellular communication in toxicology." Corriell Institute, Camden, NJ, Jan. 14, 1988.

J.E. Trosko (seminar speaker), "Epigenetic toxicology: Role of chemical modulation of intercellular communication in toxicology." Univ. of Connecticut, Storrs, Jan. 15, 1988.

J.E. Trosko (distinguished faculty lecturer), "An integrative theory of carcinogenesis: Chemical, oncogene and growth factor inhibition of intercellular communication." Medical College of Ohio, Toledo, OH, Jan. 20, 1988.

J.E. Trosko (symposium speaker), "Dysfunctional intercellular communication: Implications to the cause and cure of cancer." Tampa, FL, Jan. 25, 1988.

J.E. Trosko (seminar speaker), "Role of epigenetic mechanisms in carcinogenesis: Implications for risk assessment." Oak Ridge National Laboratory, Jan. 27, 1988.

J.E. Trosko (symposium speaker), "Role of inhibited intercellular communication in tumor promotion." Hawaii, Feb. 9-14, 1988.

J.E. Trosko (invited seminar speaker), "Chemical inhibition of gap junctional communication: Implications for toxicology". Toxicology Program, University of Wisconsin, February, 1988.

J.E. Trosko (invited seminar speaker), "Chemical tumor promoters, oncogenes and growth factors: An integrated theory of carcinogenesis". Wayne State Univ., March 9, 1988.

J.E. Trosko (invited symposium speaker), "Role of inhibited intercellular communication in carcinogenesis: Implications for risk assessment from exposure to chemicals. Bioindicators: Exposure and Effects". Oak Ridge, Tennessee, March 20-22, 1988.

J.E. Trosko (invited speaker), "Frontiers in Biological Sciences" Lecture series, "Oncogenes, growth factors and chemical tumor promoters: An integrated hypothesis involving inhibitors of gap junctional communication". Case Western Reserve University, College of Medicine, April 6, 1988.

J.E. Trosko (invited lecturer), "Chemical modulation of gap junctional intercellular communication: Implications for Toxicology". Case Western Reserve, Dept. of Environmental Health Sciences, April 7, 1988.

J.E. Trosko (invited seminar speaker), "Intercellular communication and cancer: Role of oncogenes, chemical tumor promoters and growth factors". Rosewell Park Memorial Institute, Buffalo, NY, May 4, 1988.

J.E. Trosko (invited symposium speaker), "Role of oncogenes, chemical tumor promoters and growth factors in the modulation of intercellular communication during carcinogenesis." American Oil Chemist Society Mtg., Phoenix, AZ, May 10, 1988.

J.E. Trosko (invited lecturer), "Modulation of gap junctional intercellualr communication: Pharmacological and toxicological implications". Sterling- Winthrop Research Institute, Albany, NY, May 18, 1988.

J.E. Trosko (invited seminar speaker), "Toxicological implications of chemical inhibition of gap junctional communication." Natl. Center for Toxicological Research, Jefferson, Arkansas, August 8, 1988.

J.E. Trosko (invited symposium speaker), "Mechanisms and consequences of chemical modulators of intercellular communication studied by interactive laser cytometry". Soc. Analytical Cytolcgy Intern. Symposium, Breckenridge, CO, Sept. 4-9, 1988.

J.E. Trosko (invited seminar speaker), "Influence of chemical tumor promoters, oncogenes and growth factors on gap junctional communication and carciogenesis". Univ. of Colorado Health Science Center, Sept. 8, 1988.

J.E. Trosko (invited symposium speaker), "Dietary modulators of intercellular communication in carcinogenesis and atherogenesis". American College of Nutrition Mtg., New Orleans, Sept. 16, 1988.

J.E. Trosko (invited discussant), "Role of inhibited intercellular communication in pancreatic carciogenesis". Pancreas Cancer Working Group Mtg., Bethesda, Septt. 19-20, 1988.

J.E. Trosko (invited seminar speaker), "Oncogenes, chemical tumor promoters and growth factors: An integrated theory of carcinogenesis". Univ. of Kansas, Kansas City, Oct. 26-27, 1988.

J.E. Trosko (invited symposium speaker), "Stem cell theory of cancer". 18th Conference on Toxicology - H. Armstrong Aerospace Medical Res. Lab., Dayton Ohio, Nov. 1-3, 1988.

J.E. Trosko (invited seminar speaker), Cornell University, Ithaca, New York, Nov. 15, 1988.

J.E. Trosko (invited symposium speaker), "Cell-cell communication in carcinogenesis". Mouse Liver Carcinogenesis Mtg., Austin, Texas, Nov. 31 - Dec.2, 1988.



J.E. Trosko (invited speaker), "Scientific basis for epigenetic toxicology: A possible resolution of a crisis in risk assessment models". ILSI Foundation Mtg., Bahamas, Jan. 13, 1989.

J.E. Trosko (invited seminar speaker), "Epigenetic Toxicology: The implications to risk assessment." Monsanto Chemical, St. Louis, Feb. 13, 1989.

#### VII. New Discoveries, Inventions, Patent Disclosures and Specific Applications.

During the last three years under this grant, we have: (1) validated the "FRAP analysis" technique, using the Meridian ACAS-470 instrument, to measure gap junction function; (2) developed the scrape-loading/dye transfer technique to measure gap junction function and validated and integrated the technique with flow cytometry; (3) we have also developed a normal human skin keratinocyte in vitro assay to detect chemical toxicants of the human skin; (4) developed a new conceptual paradigm, "Epigenetic Toxicology" to be integrated in biologically-based risk assessment models for humans exposed to non-genotoxic chemicals; (5) demonstrated multiple mechanisms by which these epigenetic chemicals modulate intercellular communication; and (6) applied these techniques and concepts to determine how a group of known toxicants work.

There have been no patented inventions, although, in theory, a couple might have been considered as patentable potential screening assays.

#### VIII Additional Statements Regarding State of Project.

The major implication of the findings of this grant has been a new way of looking at how many toxic chemicals induce pleiotropic diseases. In addition, the work also points to the potential of reducing large number of animals to test for the potential toxic consequences in humans. On a practical level, new in vitro technologies have been developed to screen for potential human toxicants and to study the biochemical mechanisms by which they work. To underscore the significance of the aforementioned summary, we were able to organize and pull off a major international symposium on the "toxicological implications of chemical inhibitors of gap junction function".

The "bottom-line" is that U.S. AFOSR has the scientific means to evaluate the potential risk to humans after exposure to the many chemicals it must use at all levels of its operations.